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TNO report

DV2 2005-A050

Feasibility of screening for antibiotic resistance - part II

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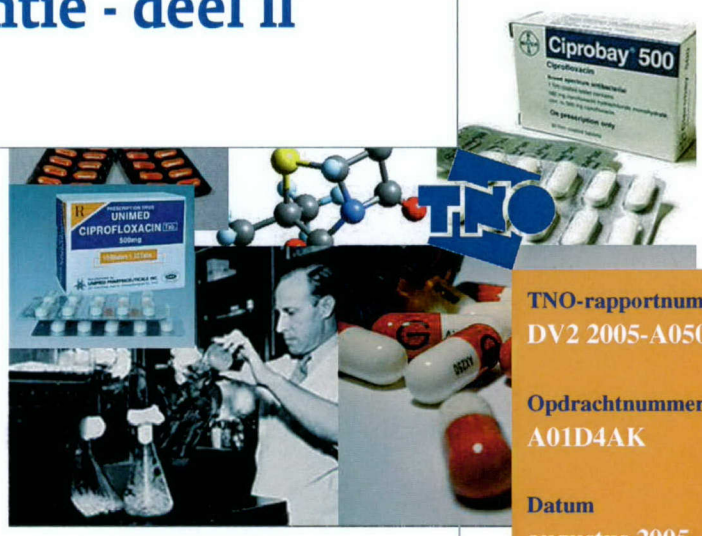
Haalbaarheid van screening op antibioticaresistentie - deel II



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In opdracht van het Ministerie van Defensie werd onderzoek gedaan naar methoden voor het aantonen van eventueel aanwezige resistentie-eigenschappen in bacteriën. Het onderzoek is uitgevoerd in de business unit 'Biologische en Chemische Bescherming' van TNO Defensie en Veiligheid. Het onderzoek is gefinancierd in het kader van programma V013 en hoort bij resultaatnummer 807a.

Probleemstelling

De huidige methodiek voor detectie van antibioticaresistentie is gebaseerd op het kweken van de bacterie, hetgeen langdurig kan zijn. Wanneer resistentie sneller herkend kan worden, kan eerder een effectieve therapie worden toegepast. Daarmee wordt tijdwinst geboekt, en mogelijk worden levens gespaard.

Beschrijving van de werkzaamheden

Er werd een theoretische studie verricht om de haalbaarheid in te schatten. Tevens werden allerlei benodigde gegevens verzameld ten behoeve van de experimentele aanpak. In de experimentele studie werden DNA-gebaseerde methoden ontworpen om twee categorieën resistentiegenen aan te tonen, waaronder ciprofloxacineresistentie. Beide methoden werden getest op kunstmatig resistent gemaakte bacteriestammen.

Resultaten en conclusies

Uit de theoretische studie blijken de voor- en nadelen van de ontworpen methoden, en ontstaat een indruk van de hoeveelheid werk om dit voor alle relevante bacteriën op te zetten. De ontworpen methoden werkten goed op de twee kunstmatig resistent gemaakte bacteriestammen. De methode blijkt in 6 uur of minder uit te voeren. Het percentage vals-negatieven is maximaal 20%, maar waarschijnlijk veel lager.

Toepasbaarheid

De methode is toepasbaar in situaties waarin de bacteriesoort reeds geïdentificeerd is. Direct daarna kan worden vastgesteld of bijvoorbeeld ciprofloxacineresistentie aanwezig is. Indien dit het geval blijkt te zijn kan kostbare tijd gewonnen worden, en kunnen mogelijk zelfs levens gered worden, door een ander antibioticum toe te passen. De methode is in de huidige vorm niet te velde toepasbaar. Snel transport van de monsters naar een laboratorium is essentieel om de beoogde tijdwinst te kunnen boeken.

Vervolgafspraken

De ontworpen methode kan nog verder worden verbeterd, en worden toegepast op een groter aantal bacteriesoorten. Met name de toepassing op Gram-positieve bacteriën (waaronder *Bacillus anthracis*) moet nog verder ontwikkeld worden. Daarnaast zijn er mogelijkheden om een mutatie-analyse te ontwerpen waarmee binnen 2 uur resultaat te behalen is. Deze mutatie-analysemethode is tevens geschikter voor gebruik te velde. Dit onderzoek zal plaatsvinden onder resultaatnummer 807b.

PROGRAMMA	PROJECT
Programmabegeleider A.S. de Koning, arts, MGFB	Projectbegeleider dr. H.J. Jansen, MGFB
Programmaleider Dr. M.S. Nieuwenhuizen, TNO Defensie en Veiligheid	Projectleider ir. M.P. Broekhuijsen, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming
Programmatitel Passieve verdediging tegen NBC-wapens	Projecttitel Feasibility antibiotica resistentie screening
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Programmaplanning Start 2001 Gereed 2005	Projectplanning Start 2001 Gereed 2003
Frequentie van overleg Met de projectbegeleider werd twee maal per jaar gesproken over de invulling en de voortgang van het onderzoek.	Projectteam ir. M.P. Broekhuijsen, W.C.M. van Dijk, A.M.B.C. de Vries en dr. M. Polhuijs, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming

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Summary

Goal
The goal of the project is to study the feasibility of developing new methods or improving existing methods for the detection of antibiotic resistance in bacteria. The aim was to focus on DNA-based methods, which are inherently faster than conventional methods and more suitable for field use.

Theoretical study
The origin of antibiotics is described, as well as the increasing problem of resistance against antibiotics. Mechanisms of action and resistance are described, the difference between acquired resistance genes and resistance mutations is explained, and examples are given. The feasibility of using Polymerase Chain Reaction (PCR) as a method for the detection of acquired resistance genes is explored, using tetracycline as example. The properties of ciprofloxacin are treated, and data on the mutations that give rise to ciprofloxacin resistance are given.

Experimental study
A PCR is developed for detection of resistance against ampicillin, tetracycline, and chloramphenicol in *Escherichia coli*. A multidrug resistant strain of *Serratia marcescens* is analysed for it's resistance against ciprofloxacin and the presence of mutations in the gyrase A gene. One strain of *S. marcescens* is made resistant against ciprofloxacin and it's mutations are analysed. PCR assays, suitable for several species, are developed to amplify a region of the gyrase A gene where mutations occur that result in resistance. Sequencing of the resulting PCR products is shown to reveal the important mutations, as expected.

Main results and conclusions
- Designing PCR assays for acquired resistance genes is relatively easy, as shown for ampicillin, chloramphenicol, and tetracycline, using *E. coli* as model. However, it is a large task for all relevant species, because of sequence heterogeneity in the corresponding genes of different species, exemplified by comparing tetracycline resistance genes.
- Resistance against ciprofloxacin is mostly caused by mutations in a limited region in a limited number of genes. In the case of Gram-negative bacteria, this is almost always the QRDR region of the *gyrA* gene, and mostly at three specific positions in the gene.
- A limited set of PCR primers has been designed, suitable for a short list of relevant bacterial species. Using this set, PCR products can be obtained in all cases. The DNA sequence of the PCR products readily shows if mutations are present.
- It proved difficult to obtain ciprofloxacin resistant strains of the relevant species for testing of the method.
- Experimental induction of ciprofloxacin resistance is possible.
- Induction of ciprofloxacin resistance in *S. marcescens* at or above a level of 4 µg/ml consistently corresponded with a specific mutation at a specific position in the *gyrA* gene, giving confidence in the usefulness of this approach.
- The success rate of the method for Gram-negative bacteria cannot be reliably estimated, but is thought to be 80% or much higher.
- The time scale of the method is estimated to be around 6.5 hours, assuming the presence of DNA sequencing equipment.

Further study and recommendations

The following recommendations are made.

- To search for and obtain (either acquire from elsewhere or induce experimentally) additional resistant strains, for further validation of the method.
- To extend the number of PCR primers for the QRDR region of the *gyrA* gene, for all relevant species.
- To design mutation assays for the QRDR region of the *gyrA* gene, which would result in a much faster method.
- To investigate existing methods for screening of MRSA strains.

Contents

Abbreviations

ampR	ampicillin resistance gene
ATCC	American Type Culture Collection
bp	base pair(s)
camR	chloramphenicol resistance gene
DNA	Deoxyribo-Nucleic Acid
dNTP	deoxy-Nucleoside-Tri-Phosphate
gyrA	gyrase A gene
LB	Luria broth
NCBI	National Center for Biotechnology Information
NCC	Netherlands Culture Collection
NCTC	National Collection of Type Cultures
PCR	Polymerase Chain Reaction
RNA	Ribo-Nucleic Acid
tetR	tetracycline resistance gene
TSA	Trypticase soy agar
QRDR	Quinolone resistance determining region

1 Introduction

1.1 Goal and approach of the project

During an encounter of biological warfare agents, several actions need to occur. Two early actions are detection and identification. Assuming that these are succesful, the local commander is aware of the threat and knows the type of agent involved. Adequate protection measures have been taken, samples are secured, and medical countermeasures are prepared. The latter can include preparing antibiotics for use if personnel has been infected after all. However, this would be useless if the threat agent is resistant to the antibiotic. To ensure effective therapy, the agent should be screened for any antibiotic resistance that it may possess.

The goal of the project is to investigate, develop, improve, or implement methods for the detection of antibiotic resistance in bacteria.

Most currently used methods for screening antibiotic resistance in a strain is by culturing in the presence of antibiotics (Figure 1). Several commercial systems exist for this purpose. These methods were not explored or evaluated. The aim was to develop DNA-based methods, which are inherently faster than conventional methods and more suitable for field use.

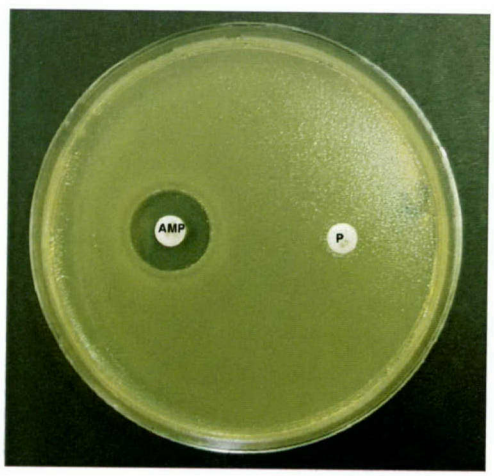

Figure 1 Example of antibiotic resistance testing using culture media. *Escherichia coli* bacteria are cultured as a layer on an agar plate. The susceptibility of the bacteria towards certain antibiotics is shown by applying a drop of antibiotic on the agar plate before culturing, resulting in a circular clear region (AMP) where bacteria will not survive. Resistant bacteria will multiply regardless of the presence of antibiotic (P). AMP: Ampicillin, P: penicillin.

The study is directed towards operationally relevant antibiotics and strains, i.e. antibiotics that are commonly used for military personnel, and bacterial species that are considered to be biowarfare agents.

1.2 Background

When encountering bacterial infections in patients or potentially pathogenic bacteria in environmental samples, the immediate need is to identify the bacteria. Once knowing the identity of the bacteria, one can prepare appropriate medical countermeasures. The immediately following concern is whether the identified bacteria carry any antibiotic resistance. Most bacterial infections can be treated effectively using antibiotic compounds, provided they are in sufficient stock and administered timely (early in the infection process). However, different bacterial species can vary in their susceptibility towards different antibiotic compounds. Even worse, many bacterial strains have developed resistance against specific antibiotics. Nowadays, many antibiotic compounds are known, both naturally occurring and of synthetic origin, which fall into several classes, based on mechanism and action. Resistance against all known antibiotics has been described.

1.2.1 Discovery of antibiotics

In 1929, Alexander Fleming (Figure 2) observed that the fungus *Penicillium notatum* was able to produce a compound that had an antibacterial effect. The compound, named Penicillin, appeared to be active against many different types of bacteria and was not toxic for man or animal. This of course is a fine example of a very beneficial property of an otherwise detrimental organism (Figure 3).

Figure 2 Alexander Fleming in his laboratory.

Figure 3 Food spoiling by the fungus *Penicillium*.

Due to the instability of the compound, it was only in 1939 that a group in Oxford (UK) headed by Howard Walter Florey and E.B. Chain was able to isolate and characterize the compound, leading to the practical use of the first and possibly most important antibiotic. The discovery of antibiotics and the development for medical use was honored by awarding the Nobelprize to Fleming, Florey, and Chain (in 1945) for penicillin, and to Selman Abraham Waksman (in 1952) for streptomycin. During the following decades, many antibiotics were discovered and developed for clinical use. Almost everyone of us has used or will use an antibiotic for curing a bacterial infection at least once in our lifetime.

1.2.2 Development of resistance

During the decades after the Second World War, it was generally believed that antibiotics were the perfect cure for bacterial infections and that mankind could stop worry about them. However, during the last couple of decades of the twentieth century, concern rose about the development of resistance. It is now generally accepted that the widespread use of antibiotics, in medicine, food, and veterinary practice, has caused the equally widespread occurrence of resistance. Insufficient hygiene in environments where many bacteria and patients are in close vicinity of each other (e.g. hospitals) further stimulated the spread of resistance (Figure 4).

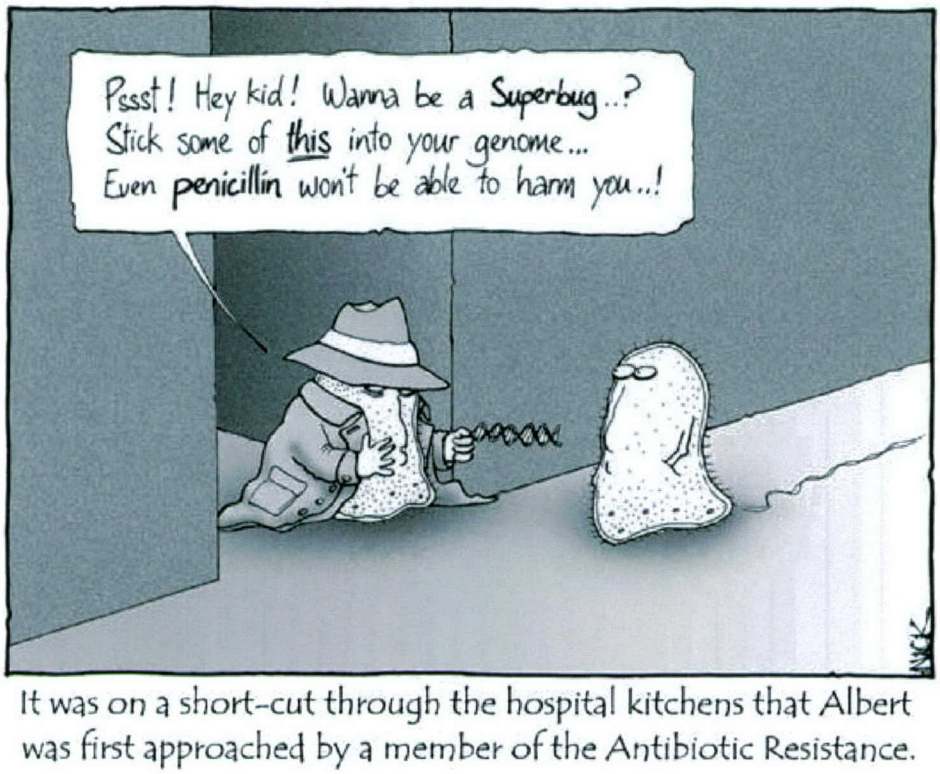

Figure 4 Spread of antibiotic resistance through insufficient hygiene in hospitals.

The development of new classes of antibiotic compounds by scientists and the following development of resistance by bacteria has taken the nature of an arms race. Nowadays, resistance has been described against every type of antibiotic compound that is known. Many multiresistant bacteria (which are resistant against different types of antibiotic compounds) have been described. The occurrence of so-called MRSA (methicillin resistant *Staphylococcus aureus*) bacteria, popularly referred to as 'hospital bacteria', has caused wide concern. Infections caused by these bacteria are more difficult to cure, or require special antibiotics that are sometimes referred to as 'last resort'. When MRSA is encountered, individual patients, or even whole departments, are quarantined, at significant cost. The occurrence of MRSA is spreading, and is becoming quite common in some countries, including Mediterranean countries. Thanks to strict measures and relatively limited use of antibiotics, it is still rare in the Netherlands. In general however, antibiotic resistance is of growing concern.

Another point to consider is the fact that resistant bacterial strains were actually developed within the former Biopreparat conglomerate in the former Soviet Union, as part of their offensive biological weapons program. The aim was to make these strains more resistant to antibiotics that were commonly used in the western countries. It is not publicly known whether any resistant strains from these sources still exist.

In Madagascar, several multiresistant strains of *Yersinia pestis* have been isolated, although it is assumed that these have naturally originated in the region (Hinnebusch et al., 2002). It illustrates, however, that biological (warfare) agents with multiple resistance characteristics can be encountered.

2 Materials and methods

2.1 Bioinformatics

Genes or DNA sequences were searched in Genbank on the NCBI website. Specific genes of interest were downloaded and analyzed using the software package DNAMAN version 5.2.9 (Lynnon Biosoft, Canada). Primers were designed using the primer-design tool in DNAMAN, with 60+/-2 °C as standard annealing temperature. DNA sequences were aligned using the Multiple Sequence Alignment tool in DNAMAN with the default parameters. Homology trees were constructed from the alignment output, using the function 'homology tree'.

2.2 Bacterial strains and lysates

The following strains were used in this study:

- *Serratia marcescens* ATCC13880 (type-strain, sensitive to antibiotics).
- *Serratia marcescens* ATCC43297 (multidrug resistant, including ciprofloxacin) (Sanders et al. 1986).
- *Escherichia coli* ATCC11775.
- *Escherichia coli* JM109.
- *Escherichia coli* XL1-blue
- *Yersinia pseudotuberculosis* ATCC29833.

Strains with an ATCC number were purchased from ATCC. The other *E. coli* strains were purchased from Stratagene. *S. marcescens* and *E. coli* strains were cultured in LB medium or on LB agar plates, at 37 °C. *Y. pseudotuberculosis* was cultured on TSA plates at 37 °C.

Bacterial thermolysates (certified killed) of *Coxiella burnetii*, *Francisella tularensis*, *Vibrio cholerae*, and *Yersinia pestis* were kindly provided by dr. Mats Forsman from the Swedish Defence Research Agency (FOI) in Umeå, Sweden.

2.3 Transformation of bacteria with resistance genes

E. coli bacteria were transformed using established methods (Sambrook et al., 1989), using plasmid pBR325. After transformation, bacteria were plated out and cultured on LC medium containing ampicillin (Sambrook et al., 1989).

2.4 PCR and gel electrophoresis

Cells from *E. coli* were used directly from the culture in the PCR. For *S. marcescens*, DNA had to isolated (see below) for PCR to be successful. PCR was performed using an MJ Research PTC-200 thermocycler, standard PCR tubes of 0.5 ml volume, with a reaction mix volume of 50 µl. Taq-polymerase and dNTP-mix was used from Roche Diagnostics. End concentrations in the PCR reaction mixture were 2.5 units Taq-polymerase, 1.5 mM MgCl2, 0.2 µM primers, 1 mM dNTP. A standard program was used with the following parameters:

1 5 min. 92 °C
2 30 sec. 92 °C

3 30 sec. 60 °C
4 30 sec. 72 °C
5 goto 2, 34 times
6 min. 72 °C
7 15 °C for ever
8 end.

After PCR, a 10 µl sample was analyzed on a standard 1.2% agarose gel in 0.5x TBE buffer (0.89 M Tris borate, 0.02 M EDTA, pH 8.3). Loading buffer was used according to Sambrook et al (1989). Electrophoresis was performed during 45 minutes at 100 mA. Gels were visualized using EtBr. Molecular weight markers used was M-9 (digest of DNA from ΦX174 with HinfI, obtained from Eurogentec), containing DNA fragments of 726, 713, 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 42, 40, 24 bp.

2.5 DNA manipulations

DNA isolation from *S. marcescens* for PCR was done using the DNA isolation kit from PureGene, according to the manufacturers instructions. For DNA sequencing, the GeneClean Spin kit was used instead, according to the manufacturers instructions. DNA sequencing was performed by the company Baseclear in Leiden, using their standard procedures. PCR amplicons and PCR primers were sent to Baseclear for sequencing. Resulting DNA sequences were received from Baseclear by email.

3 Theoretical study

The following items are addressed in this theoretical study:

3.1) Mechanism of action and resistance
For a better understanding of antibiotic susceptibility and resistance, it is necessary to distinguish between different mechanisms of action (from the antibiotic point of view) and different mechanisms of resistance (from the bacterial point of view).

3.2) Acquired resistance genes
In many cases the bacterium acquires resistance by acquiring a new gene which confers resistance. The new gene may come from other sources, e.g. another bacterial species. Molecular methods for detecting resistance based on acquired genes can simply target such genes.

3.3) Resistance by point mutations in household genes
In many other cases resistance is based on (point) mutations in genes that were already present in the bacterium, and have some particular function in the bacterium. In these cases a detection method should target only the mutation(s) rather than the presence of the whole gene.

3.4) Ciprofloxacin
According to representatives of the military medical forces, ciprofloxacin is an antibiotic used by the Dutch Armed Forces. Some aspects of ciprofloxacin are discussed, including its mechanism of action, how resistance against ciprofloxacin works, and which point mutations often occur in the gene(s) involved in resistance.

3.5) Model resistant strains
The availability of resistant strains is discussed.

Many excellent general textbooks are available on the topic of antibiotics and resistance, e.g. Walsh C (2003), Antibiotics: Actions, origins, resistance.

3.1 Mechanism of action and resistance

The mechanism of action of antibiotics and the mechanism of resistance against antibiotics are related to essential biochemical processes inside the cell, such as DNA replication or protein synthesis. An antibiotic compound interferes in an essential biochemical process, thereby killing the organism. Some of these biochemical processes, although 'universal' for all living organisms, are sufficiently different between prokaryotic organisms (including bacteria) and eukaryotic organisms (including animals). Because of this, antibiotics exist that are able to kill bacteria but are completely harmless against animals. Viruses are not susceptible to antibiotics, since they rely completely on the biochemical processes of their host.

3.1.1 Mechanism of action

Four different classes of antibiotics are generally described, based on their mechanism of action:

- Inhibition of cell wall synthesis.
- Inhibition of protein synthesis.

- Inhibition of DNA precursor synthesis.
- Inhibition of DNA or RNA synthesis.

These are all basic and essential biochemical processes in all living organisms. Inhibition generally occurs by the selective binding of the antibiotic to an essential enzyme involved in the biochemical process. Examples are shown in Figure 5.

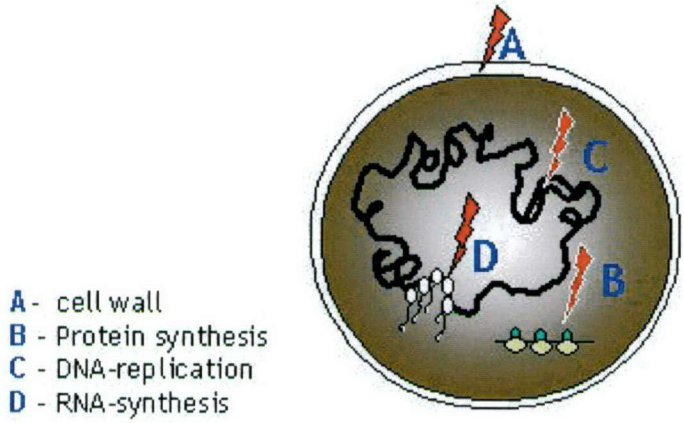

Figure 5 Examples of mechanisms of antibiotic action, aimed at different targets in a bacterial cell.

3.1.2 *Mechanism of resistance*

Several strategies exist to escape the inhibitory action of an antibiotic. These are:

- By target modification.
- By antibiotic modification.
- By antibiotic efflux pump.

Two mechanisms are shown in Figure 6. The mechanism of antibiotic modification includes the option of cleavage of the antibiotic. The efflux pump is also shown in Figure 6. Target modification is not shown in Figure 6.

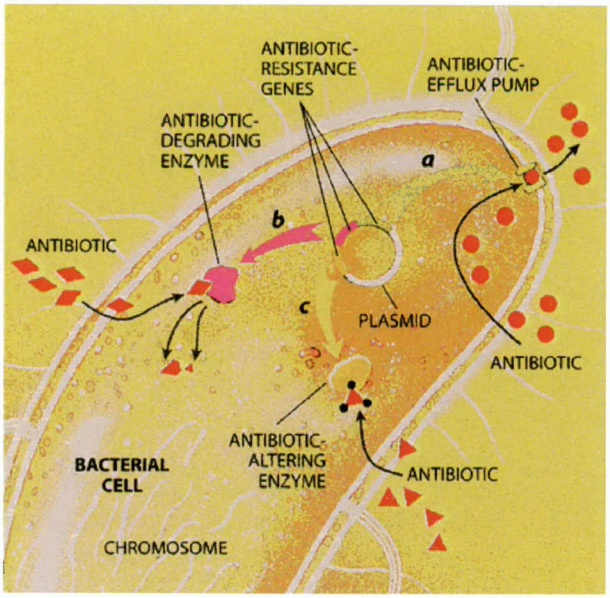

Figure 6 Mechanisms of antibiotic resistance. Only antibiotic modification and efflux pump are shown. Antibiotic target modification is not shown.

Target modification means that the target of the antibiotic, e.g. an essential enzyme, is altered (by a mutation in the encoding DNA or other alteration) resulting in the inability of the antibiotic to bind to it. As long as the modification of the target does not interfere with the normal biochemical processes in the cell, the organism has thus developed resistance. In most cases this type of resistance involves a point mutation in an otherwise normal cellular gene with a normal function.

Antibiotic modification mostly occurs by an enzymatic process that is efficient enough to render the antibiotic molecules harmless, i.e. unable to bind to their target anymore. Antibiotic modification can occur by attaching a chemical group (e.g. methyl or phosphate) or by cleavage. The enzyme responsible for this antibiotic alteration is often encoded by a so-called acquired gene, i.e. a gene that originally was not present in the cell, and does not perform any other 'normal' function.

The third strategy (antibiotic efflux pump) simply involves an efficient means of pumping the antibiotic out of the cell, faster than it gets in. This can be performed by an altered pump mechanism which is made more efficient or specific for removing the antibiotic, or by an acquired gene.

3.1.3 Examples of antibiotics

Three examples of antibiotics are given below, to illustrate the above.

Penicillin (Figure 7)
Action: Inhibition of cell wall synthesis.
Resistance: several options, a common one is inactivation of the antibiotic by β-lactamase. This enzyme is encoded by an acquired gene.

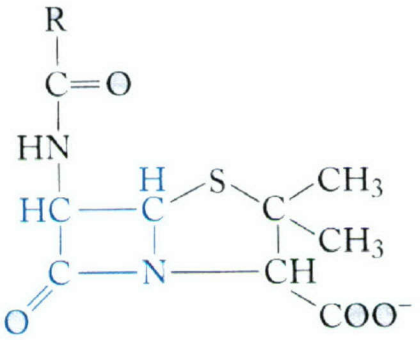

Figure 7 The molecular structure of penicillin.

Tetracycline (Figure 8)
Action: Inhibition of protein synthesis.
Resistance: several options, a common one is induction of a specific pump.

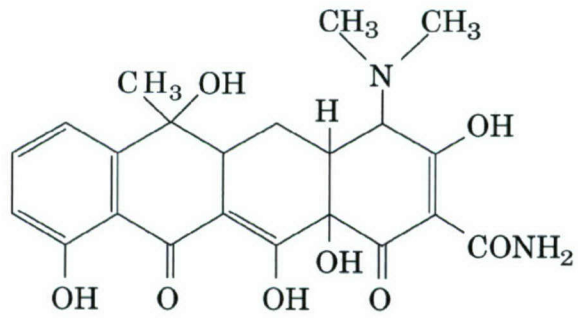

Figure 8 The molecular structure of tetracycline.

Ciprofloxacin (Figure 9)
Action: Inhibition of DNA synthesis.
Resistance: mainly target alteration, e.g. DNA-mutations in the *gyrA* gene, encoding subunit A of DNA-gyrase. Ciprofloxacin is not able to bind and inhibit the mutated DNA-gyrase, whereas the mutated enzyme is still fully functional.

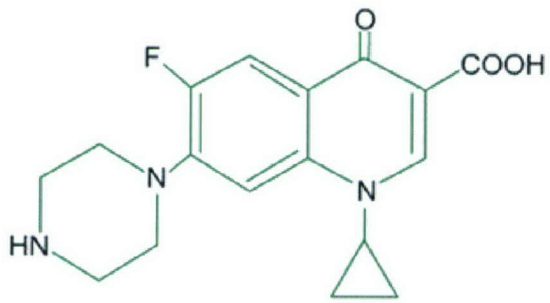

Figure 9 The molecular structure of ciprofloxacin.

3.2 Acquired resistance genes

Acquired genes are genes that were not originally present in the host organism, but acquired from another source, e.g. another related or unrelated organism. Presumably, such a gene must have originated somewhere in some organism by evolutionary mutations from another existing gene. Nevertheless, it is nowadays realized that the acquisition of genes from other organisms is widespread in nature, especially between bacteria, even completely unrelated bacteria. Although the process of acquisition itself is rare, the mind-boggling numbers of bacteria in nature ensure that it occurs quite frequently. The fact that genes can be acquired through a natural process means that a new resistance gene (or any other gene) may originate just once in nature and thereafter spread to other organisms. It is believed that this is one of the main driving forces in evolution, ever since life arose on earth.

An important practical consideration is that acquired genes are obviously not essential for the host organism, and are normally, i.e. in the original, 'wild type' organism, not present. Therefore, detection of resistance can be done by simply screening for the presence of such a gene. This can easily and efficiently be done by using PCR, provided that enough sequence data about the gene(s) is known.

3.2.1 *Examples of acquired genes and their detection*

Resistance against penicillin is caused by an acquired gene encoding the enzyme β-lactamase, which can hydrolyze penicillin, thereby rendering it inactive. The enzyme β-lactamase is not essential and normally not present in bacteria. It's only task is to hydrolyze penicillin.

Resistance against tetracycline is also caused by an acquired gene. This gene, however, encodes an efflux pump, which can efficiently remove tetracycline from the cell, without actually inactivating it. The efflux pump gene is also not essential and normally not present in bacteria.

An obvious way to detect resistance against penicillin or tetracycline would be to design a PCR assay specific for the genes involved. Normal susceptible strains do not have these genes and no PCR product would be formed, whereas analysis of resistant strains would produce a specific PCR product. Detection of resistance would thus be similar to the widely used practice of identification of bacterial species by a PCR assay specific for virulence- or other relevant genes.

3.2.2 *Case explored: tetracycline resistance*

As explained above, tetracycline resistance could theoretically be detected by a specific PCR targeted at the resistance gene. To explore this possibility, tetracycline resistance gene sequences were searched in Genbank. This resulted in many different genes from different sources (organisms). The relevant point here is whether resistance genes from different bacteria are similar enough to design a single PCR assay to be used for all, or large subsets of all. This was investigated by comparing the sequences of tetracycline resistance genes that have been found in six different bacteria:

- *Yersinia pestis.*
- *Bacillus cereus.*
- *Bacillus subtilis.*
- *Staphylococcus aureus.*
- *Escherichia coli.*
- *Serratia marcescens.*

The DNA sequences of these six genes were compared by sequence alignment, and a homology tree was constructed from the result (Figure 10).

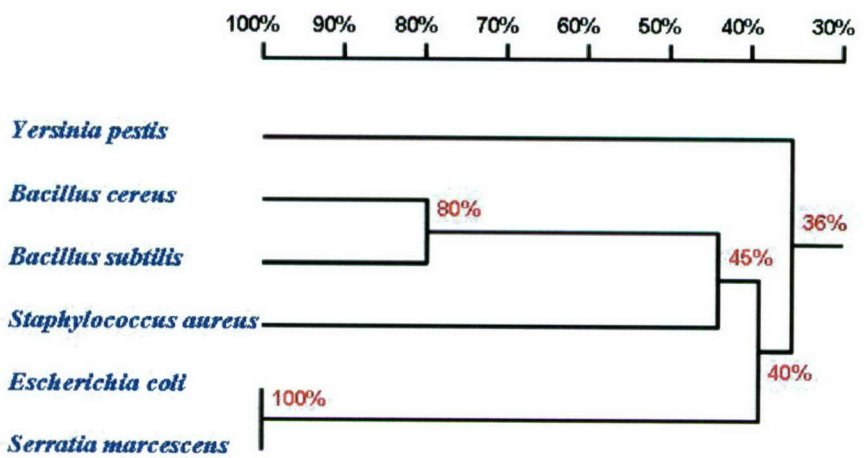

Figure 10 Homology tree of tetracycline resistance genes from six different bacteria. The distance between two bacterial species reflects genetic distance (or dissimilarity). The percentages show the level of identity in the sequence, e.g. 80% between *B. cereus* and *B. subtilis*.

The result shown in Figure 10 shows that these six genes have probably originated from at least four independent sources. The tetracycline resistance genes within *Bacillus* are clearly related. The genes in *E. coli* and *S. marcescens* must have originated from a single source and was probably obtained by one from the other, through the process of horizontal gene transfer. The other genes are probably non-related, as 40% identity is close to the obtained value when two random sequences are compared.

Of important practical consideration is the fact that there are also no significant stretches of similarity within the different DNA sequences of these genes, meaning that no single PCR assay can be designed to cover all of the genes in the above example. Instead, four different PCR assays would be required, one for the two *Bacillus* species, one for *E. coli* and *S. marcescens*, and one each for *Y. pestis* and *S. aureus*. It can be foreseen that many more separate PCR assays would be required for all relevant species. Although the design of each PCR assay would be relatively straightforward, the entire task would be significant and not attractive.

3.3 Resistance by point mutations in household genes

As explained above, resistance can also occur by target alteration. This means that the molecule (often a protein) that is targeted by the antibiotic is altered, thereby rendering the antibiotic ineffective. Often, this involves point mutations in the gene that encodes the target protein.

It is important to realize that the genes involved almost invariably perform essential functions for the host cell. As explained above, antibiotics often target essential, basic cellular functions, hence the name household genes. Point mutations in such genes are critical, and many will be fatal or have adverse effects on the host. Only a minor fraction of mutations will be neutral to the host and at the same time confer resistance to the antibiotic. It is clear that such mutations are primarily found after evolutionary pressure, i.e. after exposure to the antibiotic. Once the mutation occurs, it is stable and transmitted from the original host cell to all its progeny cells.

Molecular methods aimed at detecting resistance in these cases cannot be based on a PCR assay directed at the target gene, since the gene would also be present in a susceptible bacterium. Instead the method needs to focus on the point mutation(s). This requires that sufficient knowledge is available about:

- which genes can contain mutations.
- which area's or positions in the gene can contain mutations.
- which mutations can occur.

These facts should be known for at least several relevant bacterial species.

3.4 Ciprofloxacin

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylzuur, Figure 11) is an important antibiotic which is normally very effective and widely used.

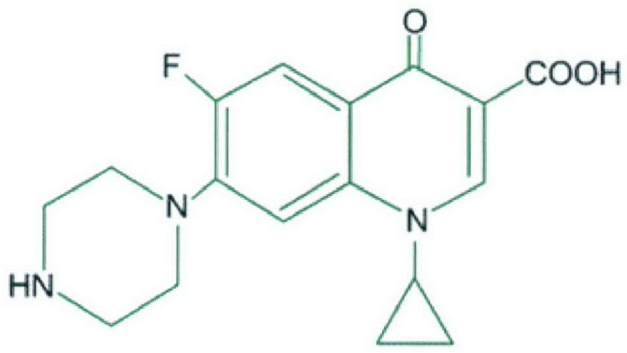

Figure 11 Molecular structure of ciprofloxacin.

Ciprofloxacin belongs to the class of fluoroquinolones, which is known since the beginning of the 1960's. From 1960 onward, four generations of fluoroquinolones were introduced on the market. The first two generations are not widely used anymore, because of widespread resistance or ineffectiveness against Gram-positive and anaerobic bacteria. The third and fourth generations, available since 1997, are more effective against Gram-positive and anaerobic bacteria and are referred to as 'expanded spectrum fluoroquinolones'.

The main target molecule of ciprofloxacin and other fluoroquinolones is the A subunit of the protein Gyrase, which is encoded by the *gyrA* gene. Gyrase is involved in the relaxation of supercoiled DNA, and is crucial for transcription and replication, both very basic functions in the cell.

In Gram-negative bacteria, host mutations that induce resistance are mostly found in a particular region of the *gyrA* gene. This region is called the Quinolone Resistance Determining Region (QRDR). Figure 12 shows the QRDR of the bacterium *Escherichia coli*. The QRDR is a short stretch of DNA in the gyrA gene, but can be represented by the corresponding amino acid stretch in the Gyrase A protein subunit, which are positions 67-106 in the case of *E. coli*. Most point mutations that render the host cell resistant are found at only three positions in the QRDR (Figure 12).

Amino acids 67-106 of gyrase A of *E. coli*

ARVVGDVIGKYHPH**G**D**S**AVY**D**TIVRMAQPFSLRYMLVDGQ

Figure 12 The QRDR of *Escherichia coli*. The region is shown as a stretch of amino acids in one-letter code, that is encoded by the QRDR of the *E. coli gyrA* gene, positions 67-106. The positions where mutations most often occur (81, 83, 87) are shown bold and underlined.

In Gram-negative bacteria, most mutations that confer resistance to ciprofloxacin are found in the *gyrA* gene encoding the Gyrase A subunit of DNA Gyrase. The *gyrA* gene belongs to a family of genes encoding so-called Topoisomerase proteins, which can alter the supercoiled structure of doublestranded DNA. Topoisomerases act by transiently cutting one or both strands of the DNA. Topoisomerase type I cuts one strand whereas Topoisomerase type II cuts both strands of the DNA to relax the coil and extend the DNA molecule. Gyrase is a type II Topoisomerase. It should be noted that

two types of Topoisomerases have been defined (type I and type II), whereas at least four specific Topoisomerase proteins are known, which are called Topoisomerase I, II, III, and IV. Topoisomerases I and III are both of type I, and Topoisomerases II and IV are both of type II.

The regulation of DNA supercoiling is essential to DNA transcription and replication, when the DNA helix must unwind to permit the proper function of the enzymatic machinery involved in these processes. Topoisomerases serve to maintain both the transcription and replication of DNA. Aside from Topoisomerases I and II, more Topoisomerases have been discovered. Topoisomerase III may regulate recombination while Topoisomerase IV regulates the process of segregating newly replicated chromosomes from one another.

Two proteins encoded by this family of genes are relevant in this study: Topoisomerase II and IV. Topoisomerase II, also known as Gyrase, consists of two different subunits (A and B), each in two copies per protein molecule (A_2B_2). The subunits are encoded by two genes, *gyrA* and *gyrB*, respectively. Topoisomerase IV likewise consists of two different subunits (A and B), each in two copies per protein molecule (A_2B_2). The subunits are encoded by two genes, *grlA* and *grlB*, respectively. The *grlA* gene is also known as *parC*, and the *grlB* gene as *parE*.

Table 1 Comparison of Topoisomerases. Topoisomerases II and IV are both type II Topoisomerases which cleave DNA by introducing doublestrand breaks. Type I Topoisomerases (Topoisomerases I and III) cleave DNA by introducing singlestrand breaks. Names are based on genes described in *E. coli*. Names can be different in other organisms.

Topoisomerase	Alternate name	Type	Genes	Protein composition
I	-	I	*topA*	
II	DNA gyrase	II	*gyrA* and *gyrB*	A_2B_2
III	-	I	*topB*	
IV	-	II	*grlA* and *grlB* (or *parC* and *parE*)	A_2B_2

Mutations that confer resistance against ciprofloxacin have been found in all four genes encoding subunits of Topoisomerase II and IV, and also in an entirely different gene encoding an efflux pump. However, in Gram-negative bacteria mutations that confer resistance to ciprofloxacin are predominantly found in *gyrA*, much less frequently in *grlA*, and rarely in other genes (Drlica and Zhao 1997). In Gram-positive bacteria mutations are predominantly found in *grlA*, less often in *gyrA*, and rarely in other genes (Drlica and Zhao 1997).

3.5 Model resistant strains

Whereas the clinical practitioner obviously is not happy when encountering a ciprofloxacin resistant strain, the research scientist is eager to get one, for use in a model system or for testing methods. During the study, it appeared to be rather difficult to obtain ciprofloxacin resistant strains. These strains are occasionally found in clinical practice, but are not routinely secured for scientific research.

Two local hospitals were contacted and asked for ciprofloxacin resistant strains. The medical microbiologists stated that no suitable strains were in stock but they were willing to supply whatever they would encounter.

Screening of the catalog of the strain collection from the American Type Culture Collection (ATCC) resulted in one pair of apparently suitable strains, i.e. a wildtype and a resistant strain of *Serratia marcescens*. These two were purchased and analyzed (see Chapter 5).

4 Experimental study

4.1 Approaches followed

The experimental study was aimed at developing DNA-based methods for detection of antibiotic resistance. Two approaches were followed:

1 PCR on acquired genes associated with some commonly encountered antibiotic resistances. This was performed on tetracycline-, ampicillin-, and chloramphenicol-resistance genes in *Escherichia coli*.
2 PCR on the QRDR region of the *gyrA* gene and sequencing of the amplicon.

The first approach was straightforward and uncomplicated. As explained in the previous chapter, this method was not expected to be universally applicable because of sequence dissimilarities in different organisms. However, the method shows that this approach is feasible when enough PCR assays would be developed for all relevant organisms.

The second approach was aimed at detecting mutations in 'household' genes that confer resistance, in this case mutations in the *gyrA* gene that confer ciprofloxacin resistance.

4.2 Resistant strains

Validation of any chosen approach depends on the availability of pairs of susceptible and resistant strains on which the designed assays should be tested. Strains which are resistant against tetracycline, ampicillin, and chloramphenicol were readily obtained. This was not surprising because resistance in these cases is dependent on acquired genes. Moreover, the genes that confer resistance against tetracycline, ampicillin, and chloramphenicol are very well known and have been used in genetic modification studies for many years (Sambrook et al., 1989). As a result, many plasmids are available containing these resistance genes. Most of these plasmids are compatible with *E. coli* and can easily be used for these studies. Resistant strains were therefore prepared by introducing the appropriate plasmids into *E. coli* bacteria. The untransformed *E. coli* strain then served as a negative control.

Ciprofloxacin resistant strains were not easily obtained. Eventually, a resistant strain was developed from *Serratia marcescens*, specifically for this study. The resulting strains were characterized (see below).

4.3 First approach: PCR on acquired genes

This approach is described in more detail in a report by Polhuijs and de Vries (DV2 2005-A060).

Three acquired resistance genes were chosen, all known to exist in *E. coli*. These were tetracycline (*tetR*), ampicillin (*ampR*), and chloramphenicol (*camR*) resistance genes. These genes have all been cloned years ago and have been used worldwide. Conveniently, all three are present in a well-known plasmid called pBR325 (accession number L08855 in Genbank).

Because designed PCR primers do not always function as expected, three primer sets were designed for each gene. The annealing temperature was chosen as 60+/-2 °C, and the amplicon sizes ranged from 200-301 bp. Although at this stage only classical PCR was used, the primers were designed to have suitable properties for use in real-time PCR (Broekhuijsen and Boomaars, TNO report PML2001-B5). Details of the designed primers are given in Annex A.

All nine primer sets were tested on purified pBR325 DNA and on crude bacterial suspensions of *E. coli* cells containing pBR325. All primer sets performed well, producing PCR products with the expected sizes, whereas the use of wildtype *E. coli* cells did not result in any PCR product (Figure 13). The expected sizes of the PCR products with primer sets FR01-06 are 201, 250, 290, 201, 251, 298 bp, respectively (Annex A). PCR products from the camR gene are not shown here.

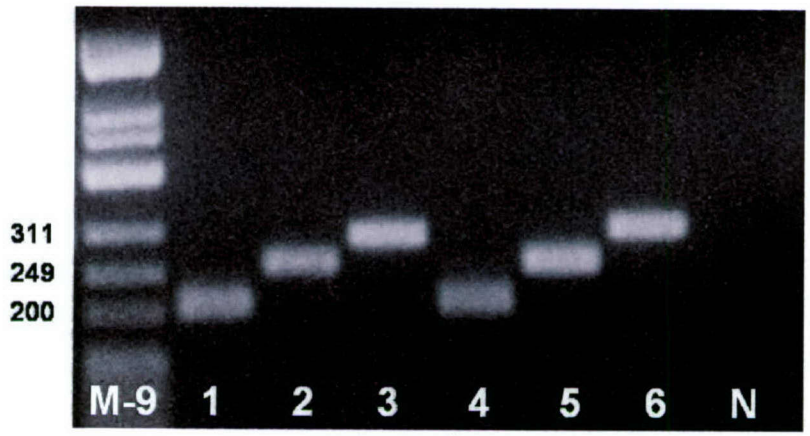

Figure 13 PCR on acquired genes. Products from PCR on crude extracts of cultures of *E. coli* bacteria transformed with plasmid pBR325 were separated by gel electrophoresis. M-9: size marker M-9, with the sizes of three fragments shown (in bp). Lanes 1-6: PCR products using primer sets FR01-06, respectively (Annex A). N: negative control (untransformed *E. coli*).

4.4 Second approach: PCR and sequencing on QRDR

For this approach only the *gyrA* gene was chosen. As explained in the previous chapter, some other genes can also be involved in ciprofloxacin resistance, but *gyrA* appears to be the most important one, especially in Gram-negative bacteria. Initially, only Gram-negative bacteria were explored.

4.4.1 Gene sequences

Nowadays, many DNA sequences have been determined and published, either from cloned single genes, or from genomic sequencing projects. The *gyrA* gene is a well-known gene, and it's sequence is published for a number of relevant bacteria. A search was performed in Genbank and *gyrA* gene-sequences were downloaded and compared. For convenience, the rather long gene-sequence (2628 bp for *E. coli*) was not used entirely, but only the first 420 bp (encoding the first 140 amino acids). This region covers the QRDR region and flanking sequences, and is long enough to design PCR primers.

Sequences of *gyrA* were downloaded for the following 19 Gram-negative bacteria:

Brucella melitensis, Brucella suis, Citrobacter freundii, Coxiella burnetii, Enterobacter aerogenes, Erwinia carotovora, Escherichia coli K12, Francisella tularensis, Klebsiella pneumoniae, Neisseria gonorrhoeae, Providencia stuartii, Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens, Shigella flexneri, Vibrio cholerae, Vibrio parahaemolyticus, Yersinia pestis, Yersinia pseudotuberculosis. This collection represents a wide diversity within the Gram-negative bacteria. All DNA sequences were truncated to the first 420 bp and translated into protein sequences (140 amino acids). Multiple sequence alignment was done for both DNA and protein sequences. This resulted in 83% average identity level in DNA sequences and 89% average identity level in the protein sequences, reflecting the conserved homology of this gene throughout evolution.

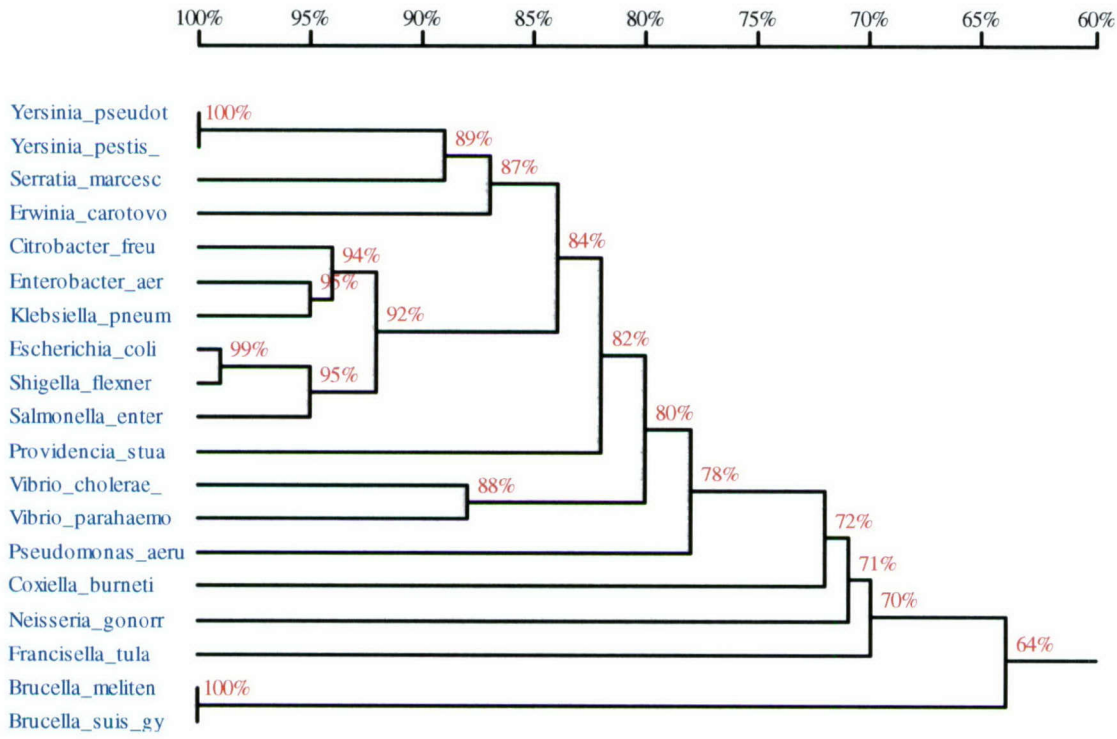

Figure 14 Homology tree of 19 gyrA sequences after alignment.

However, individual identity levels range between 64% (between *Brucella* and *Francisella*) and 100% (e.g. between the two *Brucella's*), as shown in Figure 14. Moreover, it appeared impossible to find stretches of homologous sequences long enough to design primers that are suitable for all species. Several primer-pairs had to be designed. However, attempts were made to design primers such that they would be 'partially universal', i.e. compatible with a subset of species as large as possible.

4.4.2 *Primer design and PCR analysis*

Primers were designed for amplification of the QRDR region of the *gyrA* gene of several Gram-negative bacteria, with the aim to be as 'universal' as possible. An example is demonstrated here using a subset of 5 *gyrA* sequences, derived from *Coxiella burnetii*, *Serratia marcescens*, *Vibrio cholerae*, *Yersinia pestis*, and *Yersinia pseudotuberculosis*. A homology tree is shown in Figure 15. The identity levels for this subset range between 70% and 100%, with two intermediate values close to 80% and 90%. Using this subset can show how easy, or how difficult, it is to design common primers, i.e. primers that work on all sequences.

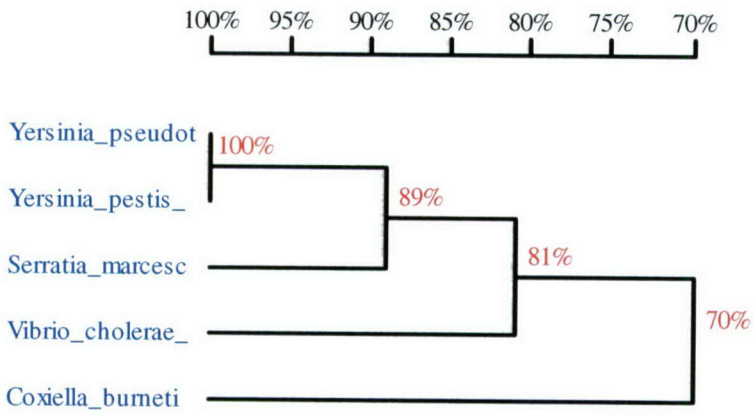

Figure 15 Homology tree of a subset of 5 gyrA sequences after alignment.

The alignment of the subset of 5 sequences was used to design primers that would be suitable for as many of the sequences as possible. The software package does not have an option for this approach, so this was done without the help of computer software. An important consideration was to focus on the 3'-end of the primer, which is the most critical. Mismatches at the 3'-end would be worse than mismatches further to the 5'-end of the primer.

Figure 16 shows the sequence alignment and the first primer set that was designed, forward primer gyrA-F01 and reverse primer gyrA-R01. Primer gyrA-F01 is a so-called degenerate primer, with two ambiguous positions (position 3 = C or G; position 12 = C or T). Primer gyrA-F01 has a perfect match with the *Yersinia's* and *Serratia*, a nearly perfect match with *Vibrio*, and a poor match with *Coxiella*. Primer gyrA-R01 has a good or perfect match with the *Yersinia's*, *Serratia*, and *Vibrio*, and a lesser match with *Coxiella*.

```
Yersinia_pseudot AGGAAGAGCTGAAAAGCTCCTATCTGGATTATGCGATGTC      80
Yersinia_pestis_ AGGAAGAGCTGAAAAGCTCCTATCTGGATTATGCGATGTC      80
Serratia_marcesc AaGAcGAGtTGAAAAaCTCgTATCTGGAcTATGCGATGTC      80
Vibrio_cholerae_ AaGAcGAGCTacgcAGtTCgTATtTGGAcTAcGCGATGTC      80
Coxiella_burneti AaGAAGAaCTcAAgcaaTCgTAcCTcGATTATGCGATGag      80
Consensus        a ga ga  t       tc ta  t ga ta gcgatg
gyrA-F01                         TCSTATCTGGAYTATGCGATGTC

Yersinia_pseudot CGTTATTGTCGGACGTGCGTTACCAGATGTCCGTGATGGA    120
Yersinia_pestis_ CGTTATTGTCGGACGTGCGTTACCAGATGTCCGTGATGGA    120
Serratia_marcesc CGTTATTGTCGGACGTGCccTgCCAGATGTtCGTGATGGA    120
Vibrio_cholerae_ gGTTATcGTgGGtCGTGCtcTtCCtGATGTgCGTGATGGc    120
Coxiella_burneti tGTTATcGTaGGgCGTGCGcTgCCtGATGTgCGTGATGGg    120
Consensus         gttat gt gg cgtgc  t cc gatgt cgtgatgg

..............

Yersinia_pseudot TAACTTCGGTTCCGTCGATGGTGACTCCGCCGCGGCGATG    360
Yersinia_pestis_ TAACTTCGGTTCCGTCGATGGTGACTCCGCCGCGGCGATG    360
Serratia_marcesc TAACTTCGGcTCCGTCGAcGGcGACTCCGCgGCGGCGATG    360
Vibrio_cholerae_ TAACTTtGGcTCgaTCGAcGGcGACTCCGCgGCGGCaATG    360
Coxiella_burneti TAACTTtGGTTCtGTaGATGGgGAtgCgcCgGCaGCcATG    360
Consensus        taactt gg tc  t ga gg ga  c  c gc gc atg
gyrA-R01                                          ATG

Yersinia_pseudot CGTTATACCGAAATCCGTATGTCTAAAATTGCTCACGAAT    400
Yersinia_pestis_ CGTTATACCGAAATCCGTATGTCTAAAATTGCTCACGAAT    400
Serratia_marcesc CGTTATACCGAAgTgCGcATGTCcAAgATTGCTCACGAAc    400
Vibrio_cholerae_ CGTTATACCGAAgTtCGTATGTCgAAAATcGCgCACGAAc    400
Coxiella_burneti CGTTAcACCGAgATtCGatTaTCccgctTTGCgCAtGctT    400
Consensus        cgtta accga  t cg  t tc     t gc ca g
gyrA-R01         CGTTATACCGAAGTGCG
```

Figure 16 Alignment of a subset of five DNA sequences. The positions of forward primer gyrA-F01 and reverse primer gyrA-R01 are shown in green. Identical nucleotides in the gene-sequences are marked yellow. Ambiguity codes: S = C or G, Y = C or T. The location numbering is shown on the right. The intermediate part of the sequence between positions 120 and 340 is left out for clarity.

The primer set gyrA-F01/R01 was used in a PCR reaction on DNA of the 5 species mentioned. Interestingly, a product of 320 bp was formed in all cases (not shown here), even with *Coxiella burnetii*, although not as efficient. This result illustrates that one primer set can indeed be designed for use with several species.

Variations of the first primer set were designed, resulting in the primers shown in Table 2. These primers were tested with 7 species in several combinations of forward and reverse primers. At least one suitable combination was found for each species (Table 3). Forward primers gyrA-F02 and -F03 are in fact redundant in this short list. The overall result is thus that 2 forward primers and 3 reverse primers together were sufficient to produce amplicons with 7 species tested.

Table 2 Primer sets designed for amplification of *gyrA* sequences. Ambiguity codes: S = C or G, Y = C or T.

Primer name	Sequence (5'-3')	Length (bases)
gyrA-F01	TCSTATCTGGAYTATGCGATGTC	23
gyrA-F02	TCTTATCTGGATTATGCGATGTC	23
gyrA-F03	TCGTATTTGGATTATGCGATGTC	23
gyrA-F04	AATTGTTGGTCGTGCTTTGC	20
gyrA-R01	CGCACTTCGGTATAACGCAT	20
gyrA-R02	CGGATTTCCGTATAACGCAT	20
gyrA-R03	CGAATTTCGGTATAACGCAT	20

Table 3 Species tested and primer sets that produced amplicons of the expected size.

Species	Forward primer	Reverse primer	Amplicon size (bp)
Coxiella burnetii	gyrA-F01	gyrA-R01	320
Escherichia coli	gyrA-F01, -02, -03	gyrA-R02	320
Francisella tularensis	gyrA-F04	gyrA-R03	294
Serratia marcescens	gyrA-F01	gyrA-R01	320
Vibrio cholerae	gyrA-F01	gyrA-R01	320
Yersinia pestis	gyrA-F01	gyrA-R01, -3	320
Yersinia pseudotuberculosis	gyrA-F01	gyrA-R01, -3	320

4.4.3 *Ciprofloxacin resistant strains*

Attempts to obtain ciprofloxacin resistant strains from two local hospitals failed, although the local medical microbiologists were willing to supply whatever they encountered. In practice, this did not result in any strain from these sources after two years.

The only set of sensitive and resistant strains that could be obtained was found in the catalog of the strain collection from the American Type Culture Collection (ATCC), i.e. a wildtype strain (ATCC13880) and a resistant strain (ATCC43297) of *Serratia marcescens*. These two were purchased and analyzed.

The *S. marcescens* ATCC43297 strain is known as a multidrug resistant strain, and is resistant against amikacin, aztreonam, carbenicillin, cefoperazone, cefotaxime, cefoxitin, chloramphenicol, ciprofloxacin, mezlocillin, piperacillin, tetracycline, and tobramycin (Sanders et al, 1986). When analyzed however, the level of ciprofloxacin resistance appeared to be rather low (0.125 µg/ml) for this strain, compared to 0.03 µg/ml for the wildtype strain ATCC13880, as shown in Figure 17. It was anticipated that this low resistance level would not be caused by a mutation in the *gyrA* gene, which was indeed shown later on (see below).

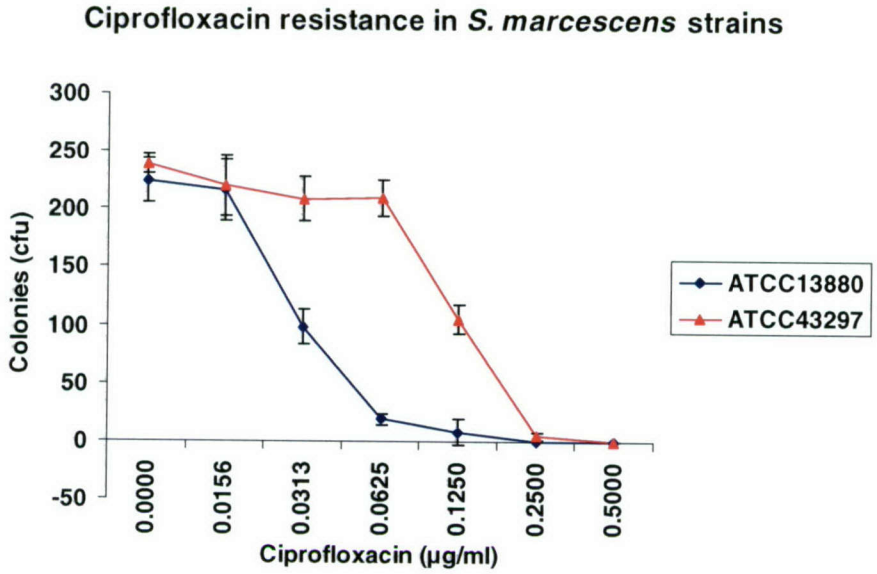

Figure 17 Resistance levels of two *Serratia marcescens* strains obtained from ATCC. Approximately 200 cfu were plated out in the presence of varying concentrations of ciprofloxacin, and surviving colonies were counted. The inhibitory concentration of ciprofloxacin is estimated at the 50% level of surviving colonies.

To obtain a mutant strain with a significant level of resistance it was attempted to induce resistance in *E. coli* JM109. PCR analysis on supposedly resistant colonies failed, or produced too little amplicon for sequencing. Eventually, the colonies stopped growing and were very difficult to culture on fresh medium. Induction of resistance in *E. coli* JM109 by this method thus proved unsuccessful.

The next attempt was done with *S. marcescens*. Induction of resistance in this species proved to be successful. Several strains were obtained with different levels of resistance (0.5, 1, 4, 8, 16 µg/ml). The higher levels would be expected to be relevant and possibly be correlated with mutations in the QRDR region of the *gyrA* gene.

4.4.4 *DNA sequencing*

First, several sensitive and/or resistant strains of *E. coli* and *S. marcescens* were analyzed with PCR, using primers gyrA-F01, gyrA-R01, and gyrA-R02 (Table 2 and 3). Amplicons of the expected sizes were produced in all cases. The resulting amplicons were used for sequencing with the same primers as used for PCR. Sequencing was done with both primers (on both strands), to obtain reliable and complete sequences.

The strains of *E. coli* used were ATCC11775, XL1-blue, and JM109. These three strains are all sensitive to ciprofloxacin (0.04 µg/ml is sufficient to kill all three strains of *E. coli*). No point mutations were found in *E. coli* ATCC11775 (wildtype strain). Several point mutations were found in *E. coli* strains XL1-blue and JM109. Most point mutations were at the third position in the codons, and did not result in a mutated amino acid. However, one mutation did result in an amino acid change, in both strains. This change was aspartate (D or Asp) to asparagine (N or Asn) at position 87 (Table 4).

Table 4 Point mutations in the QRDR region of the *gyrA* gene of two *E. coli* strains, compared to the wildtype strain ATCC11775. Point mutations are marked yellow.

Strain	ATCC11775		XL1 blue		JM109	
Amino acid position	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid
32	CGT	R	CGC	R	CGT	R
85	GTT	V	GTC	V	GTC	V
87	GAC	D	AAC	N	AAC	N
91	CGT	R	CGC	R	CGC	R
100	TAC	Y	TAT	Y	TAT	Y
111	TCC	S	TCT	S	TCT	S

As expected, no point mutations were found in the two purchased strains of *S marcescens*, the wildtype strain ATCC13880 and the multiresistant strain ATCC43297 (see above). *S. marcescens* has exactly the same amino acid sequence in the QRDR region as wildtype *E. coli* ATCC11775.

The experimentally obtained resistant strains of *S. marcescens* showed a differential result. No point mutations were found in *S. marcescens* strains with ciprofloxacin resistance at a level of 0.5 µg/ml or below. However, *S. marcescens* strains with ciprofloxacin resistance at or above a level of 4 µg/ml all had a point mutation at position 83 in QRDR, with amino acid serine (S or Ser) changed in arginine (R or Arg) (Table 5). No other point mutations were found.

Table 5 Sequenced clones of *S. marcescens* with various resistance levels. The higher levels of resistance correlated with a mutation of S (serine) to R (arginine) at position 83 in the gyrase A protein sequence.

Originating strain	Ciprofloxacin resistance level (µg/ml)	Number of clones sequenced	Mutation in QRDR of gyrase A protein
ATCC13380	0	1	none
ATCC43297	0.1	1	none
ATCC43297	0.5	1	none
ATCC43297	4	6	83S-R
ATCC43297	8	6	83S-R
ATCC43297	16	2	83S-R

5 Discussion and conclusions

5.1 Resistant strains

The availability of resistant strains as model is absolutely necessary for validating methods. Effort was put into obtaining resistant strains by purchasing from commercial sources, through contacts with local hospitals and other institutes, or by experimental induction of resistance.

5.1.1 Acquired genes

Strains of *E. coli* resistant to ampicillin, tetracycline, and chloramphenicol were easily obtained. The genetic changes that cause resistance in these cases all exist in the form of acquired genes. Moreover, in these cases well characterized resistance genes are widely available on commercially obtainable plasmids, used in genetic modification studies. The easiest way to get resistant strains was to transform sensitive *E. coli* strains with these plasmids (most available plasmids are based on *E. coli* replication origins). This is based on well established recombinant DNA methods (Sambrook et al., 1989). The obvious choice was a plasmid called pBR325 which contains all three resistance genes (*ampR*, *tetR*, and *camR*). Strains transformed with this plasmid have to be maintained on culture medium containing one of the antibiotics in order to keep the strain stably transformed. Culturing without antibiotic could eventually result in loss of the plasmid. Culturing with antibiotic was not a drawback in establishing screening methods.

Development of PCR assays for detecting these gene sequences appeared to be relatively easy. In fact, all designed primer sets resulted in the expected PCR products. The results showed that this approach for acquired genes is feasible, at least if the gene sequences involved are known.

However, the drawback of this approach is explained in the theoretical study. The gene sequences that cause resistance for these antibiotics are very heterogeneous and exist in many different bacterial species, probably through several independent evolutionary events. The original thought that one, or very few, PCR assays could suffice for an acquired resistance gene seems not to be valid. Rather, for each group of related bacterial species or genus, a separate PCR assay should be designed. Moreover, not all gene sequences for acquired genes in all relevant species are known.

Another argument is that these acquired genes do not confer the most interesting resistance properties. This is particularly true for ampicillin and chloramphenicol. Tetracycline does have some relevance and could be considered for further attempts to develop PCR assays for additional species or genus.

5.1.2 Ciprofloxacin resistant strains

A survey of strain collections in 2002 did not result in strains that seemed suitable. The collections that were searched were the American Type Culture Collection (ATCC), the National Collection of Type Cultures in the UK (NCTC), and the Netherlands Culture Collection (NCC). Only ATCC contained a set of potentially interesting strains, i.e. two *S. marcescens* strains where one was a wildtype (sensitive) strain and the other a ciprofloxacin resistant strain. The resistant strain appeared to have a low resistance

level, which was later shown to be consistent with the fact that no mutations were found in the QRDR region of the *gyrA* gene.

Contacts with local hospitals did not result in any resistant strain. Occasional phone calls did not result in any progress. Whether no strains were encountered, or that no strains were secured, is unclear. The only way to obtain resistant strains seemed to be to induce resistance experimentally. This proved unsuccessful with *E. coli*. A possible explanation for this is that the procedure was carried out too quickly, so that the bacteria were not able to escape death by point mutations. Another explanation could be that not enough live bacteria were present at the moment of challenge to allow the chance that a suitable mutation was present in the population.

The experimental induction of ciprofloxacin resistance in *S. marcescens* was successful. It should be possible to repeat this, with the purpose of obtaining resistant strains of other species for testing of screening methods.

5.2 PCR amplification

PCR primers for the QRDR region in the gyrA gene were designed for several Gram-negative bacteria. This was quite successful, resulting in a limited set of primers suitable for a number of species, thus limiting the need of designing separate primers for each species. Amplification with these primers is straightforward, resulting in clear amplification products, suitable for sequencing. The DNA sequence of these amplicons then shows if mutations are present that are expected to confer resistance to ciprofloxacin.

Design of additional primers for the relevant species should be feasible, resulting in a limited list of primers for all relevant species of bacterial warfare agents.

5.3 Sequencing of amplicons

Sequencing of amplicons is a routine method. The sequencing is performed with the same set of primers as used for the PCR, thereby ensuring reliability of the sequence, and reducing the need for additional primers specific for sequencing.

The sequencing of amplicons was also used in the study for analyzing the ciprofloxacin resistant strains. Unexpectedly, two strains of *E. coli* that are not resistant to ciprofloxacin, did have a point mutation (87D-N). Although position 87 of *gyrA* is known to be involved in ciprofloxacin resistance, the change from D to N is not dramatic, in the sense that the amino acids D and N are quite similar in structure and nature. This would explain why this point mutation in *E. coli* strains XL1-blue and JM109 does not result in resistance.

The sequencing of the experimentally obtained ciprofloxacin resistant *S. marcescens* strains showed consistent results. All analyzed strains with a resistance level at or above 4 μg/ml had a mutation, at the same position, which is known to be a 'hot spot'. This result gives confidence in the reliability and usefulness of the method.

5.4 Time scale of method

The time required for detecting ciprofloxacin resistance in a strain can be estimated. In a typical situation, one would first aim at identifying the species of a potential pathogenic organism. If a bacterial species of the list of biowarfare agents is identified, the PCR assay for identifying ciprofloxacin resistance could immediately be started, using the short list of PCR primers that is needed for this. After the PCR amplification reaction, the resulting product could be split, one part could be analyzed on a gel or stored for later analysis, the other part could be purified for sequencing.

Sequencing could be done within the same lab if equipment is available. Otherwise this should be done by external parties.

Rough estimate of time:

Cipro-PCR setup	15 min
Cipro-PCR reaction	2 h
PCR product purification	45 min
Sequencing	3 h*
Interpretation	30 min
Total time required	6.5 h

* If sequencing is performed by others, then this could result in delay of several hours to 1 or 2 days.

The analysis can be done faster with another type of assay, explained below in Further research.

5.5 Success rate of method

As explained above, ciprofloxacin resistance is mostly caused by mutations in the QRDR region of the *gyrA* gene, at particular positions, at least in Gram-negative bacteria. However, exceptions occur, and this means that finding a wildtype sequence in the QRDR region does not guarantee that the strain analyzed is susceptible to ciprofloxacin. The usefulness of the method lies in the fact that in the majority of cases, resistant strains will be easily recognized, and faster than with current methods.

It is difficult to estimate the success rate (the percentage of cases where resistance is caused by a mutation at the expected positions), but 80% would be a conservative estimate. In practice, it could be much higher.

5.6 Summary of conclusions

The most relevant statements and conclusions are summarized below.

- Antibiotics where resistance is caused by acquired genes are not the most relevant antibiotics. Examples are ampicillin (penicillin), chloramphenicol, and tetracycline.
- Designing PCR assays for these acquired resistance genes is relatively easy, as shown for ampicillin, chloramphenicol, and tetracycline, using *E. coli* as model. However, it is a large task for all relevant species, because of sequence heterogeneity in the corresponding genes of different species, exemplified by the tetracycline resistance gene.

- Ciprofloxacin is a very important antibiotic and is the first choice to treat e.g. anthrax infections.
- Resistance against ciprofloxacin is mostly caused by mutations in a limited region in a limited number of genes. In the case of Gram-negative bacteria, this is almost always the QRDR region of the gyrA gene, and mostly at 3 specific positions in the gene.
- A limited set of PCR primers has been designed, suitable for a short list of 7 relevant, all Gram-negative bacterial species. Using this set, PCR products can be obtained in all cases.
- The DNA sequence of the PCR products readily shows if mutations are present.
- It is difficult to obtain ciprofloxacin resistant strains of the relevant species for testing of the method.
- Experimental induction of ciprofloxacin resistance is possible, as shown with *S. marcescens*.
- Induction of ciprofloxacin resistance in *S. marcescens* at or above a level of 4 µg/ml consistently corresponded with a specific mutation at a specific position in the *gyrA* gene, giving confidence in the usefulness of this approach.
- The success rate of the method for Gram-negative bacteria cannot be reliably estimated, but is thought to be 80% or much more.
- The time scale of the method is estimated to be around 6.5 hours, assuming the presence of DNA sequencing equipment.

6 Further research and recommendations

6.1 Further research

Further research can be based on the results described in this report. Obviously, additional primers need to be designed for some species on the list that are not yet covered with the current primer sets, including Gram-positive species.

Resistant strains remain essential for validating the method. Resistant strains of additional species should be acquired or induced experimentally. This should at least be undertaken with a Gram-positive bacterium, e.g. *Bacillus*.

The time scale of the method is still relatively long and should be reduced further. This is theoretically possible by designing mutation assays using real-time PCR. A recent paper from Lindler (2001) shows the feasibility of this approach. Mutation assays on the *gyrA* gene of Gram-negative and Gram-positive bacteria can be designed. It is expected that this will result in a time scale of less than 2 hours.

Resistance against other antibiotics and/or resistance in other pathogens can be considered as important topics for further research. For example, the widespread occurrence of MRSA strains seems quite relevant for military operations, especially in area's abroad.

6.2 Recommendations

The following recommendations are made.

- To search for and obtain (either acquire from elsewhere or induce experimentally) additional resistant strains, for further validation of the method.
- To extend the number of PCR primers for the QRDR region of the gyrA gene, for all relevant species.
- To design mutation assays for the QRDR region of the gyrA gene, which would result in a much faster method.
- To investigate existing methods for screening of MRSA strains.

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8 Signature

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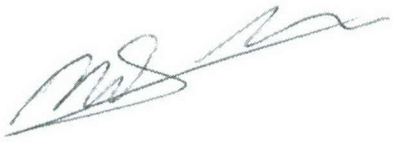

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A PCR primers for acquired resistance genes in *E. coli*.

Sequences for the tetracycline (*tetR*), ampicillin (*ampR*), and chloramphenicol (*camR*) resistance genes are taken from plasmid pBR325, accession number L08855 in Genbank (NCBI website).

Table A.1 Primer data. Strand and Position refer to the pBR325 sequence.

Primer name	Primer sequence (5'-3')	Target gene	Strand	Position
F01	GCTTGGTTATGCCGGTACTG	tetR	+	150
R01	TCGCGTAGTCGATAGTGGCT	tetR	-	350
F02	GGTTGCTGGCGCCTATATC	tetR	+	427
R02	TGAAGGCTCTCAAGGGCA	tetR	-	676
F03	GCTGCTAGCGCTATATGCGT	tetR	+	226
R03	TACCCACGCCGAAACAAG	tetR	-	515
F04	TCCATAGTTGCCTGACTCCC	ampR	+	3350
R04	GCAACAATTAATAGACTGGATGGAG	ampR	-	3550
F05	ACGGGAGGGCTTACCATCT	ampR	+	3391
R05	CAAACGACGAGCGTGACA	ampR	-	3641
F06	CGTTTGGTATGGCTTCATTCA	ampR	+	3636
R06	TGTGGCGCGGTATTATCC	ampR	-	3933
F07	TTTGAGGCATTTCAGTCAGTTG	camR	+	4629
R07	AACACTATCCCATATCACCAGCTC	camR	-	4829
F08	CAGACCGTTCAGCTGGATATTAC	camR	+	4668
R08	GTAGAAACTGCCGGAAATCGT	camR	-	4917
F09	GCTCTGGAGTGAATACCACGA	camR	+	4877
R09	CATTCTGCCGACATGGAAG	camR	-	5177

Table A.2 Amplicon sizes.

Primer set	Target gene	Amplicon size (bp)
FR01	tetR	201
FR02	tetR	250
FR03	tetR	290
FR04	ampR	201
FR05	ampR	251
FR06	ampR	298
FR07	camR	201
FR08	camR	250
FR09	camR	301

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Possibilities for screening antibiotic resistance were investigated. PCR on acquired resistance genes was shown to be possible, but a large task to develop. Mutation analysis (using PCR and DNA sequencing) in the case of ciprofloxacin resistance was shown to be effective. This approach was developed for several strains. One strain was made resistant to validate the method.

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